

REMARKS

The present application relates hybrid maize plant and seed 38J54. Applicant respectfully requests consideration of the following remarks.

Detailed Action***A. Status of the Application***

Applicant acknowledges the objection to the specification for containing blank lines in place of ATCC accession numbers on page 7 is withdrawn. Applicant further acknowledges the objection to claim 6, 12, 16, 25, and 29 are withdrawn in light of the claim amendments. The rejection of claims 1-32 under the judicially created doctrine of obviousness-type double patenting is acknowledged as withdrawn. Applicant further acknowledges the rejections of claims 1-32 under 35 U.S.C. § 102(e)/103(a) are withdrawn.

B. Claims and Specification

Applicant acknowledges the addition of new claims 41-61 placed in the format suggested by the claims faxed by Supervisory Patent Examiner Amy Nelson on August 2, 2002 and again on November 15, 2002 by Examiner David Fox. The new claims do not add new matter as there is literal support for the claims in the originally filed specification (pages 29-41, specification). Finally, Applicant submits that the Deposits section has been amended in order to properly include both the hybrid maize plant 38J54 and the inbred parents GE500988 and GE533415 within the deposit paragraph. The changes do not add new matter as there is literal support for the minor changes on page 7 in the originally filed specification. The specification has now been amended to correct these minor changes.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claim 6, 8, 11, 15, 19, 21, 28 and 32 remain rejected and new claims 33, 34, and 38-40 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. This rejection is repeated for reasons of record in the Office Action mailed July 29, 2002.

Claims 11, 15, 19, 24, 28, and 32 remain indefinite as the Examiner states the specification does not teach that the traits within Tables 1-4 can be described in the manner in the

claims. The Examiner further states the specification does not define the separation from excellent yield potential, for example, from good yield potential.

Applicant has cancelled claims 11, 15, 19, 24, 28 and 32, thus alleviating this rejection. Applicant further acknowledges the addition of new claims 41-61, placed in the format suggested by the claims faxed by Supervisory Patent Examiner Amy Nelson on August 2, 2002 and again on November 15, 2002 by Examiner David Fox as aforementioned. The new claims do not add new matter as there is support for the claims in the originally filed specification (pages 29-41, specification).

The Examiner rejects claim 6 for improper antecedent basis for the phrase "protoplasts" in line 1.

Applicant has now amended claim 5 to read --a tissue culture of regenerable cells or protoplasts--, thereby alleviating the rejection to claim 6.

Claims 8 and 21 remain indefinite for the recitation "manipulated to be male sterile". The Examiner states it is not clear if the claim is directed towards detasseled plants, or plants that have been transformed with a gene conferring male sterility.

Applicant respectfully traverses this rejection. Applicant submits support can be found on page 12 of the specification, wherein it states "[i]t should be understood that the inbred can, through routine manipulation of cytoplasmic or other factors, be produced in male-sterile form. Such embodiments are also contemplated within the scope of the present claims." Further, the specification states "hybrid maize seed is typically produced by a male sterility system incorporated manual or mechanical detasseling" (page 2, specification). In addition, the "detasseling process can be avoided by using cytoplasmic male-sterile inbreds" (page 2, specification). As taught in the specification, there are several methods of conferring male sterility. Therefore Applicant asserts that one skilled in the art would not find the terminology indefinite. However in an effort to expedite prosecution Applicant has now amended claim 8 to read --comprises a genetic factor conferring male sterility--. In addition, claim 21 has now been cancelled, alleviating this rejection.

The Examiner rejects claims 11, 15, 19, 24, 28, 32, 38, and 39 for the recitation "has derived at least 50% of its alleles" in claims 11, 15, 19, 24, 28, and 32, and "deriving at least 50% of its alleles" in claims 38 and 39 which render the claims indefinite. The Examiner states it is not clear what is meant by "derived" and "deriving".

Applicant has canceled claims 11, 15, 19, 24, 28, 32, 38, and 39, thus alleviating this rejection.

The Examiner rejects claim 33 for the recitation "a hybrid maize plant" in line 4 renders the claim indefinite. The Examiner states the claim does not clearly indicate that the hybrid maize plant in the recitation is the same as 38J54, mentioned in line 1.

Applicant has now amended the claim to replace "a" with --said--, as suggested by the Examiner, thereby alleviating this rejection.

Claim 34 stands rejected for the recitation "essentially" in line 3 which renders the claim indefinite. The Examiner continues by stating that the recitation makes the metes and bounds of the claim unclear.

Applicant has canceled claim 34, thus alleviating this rejection.

Claim 38 stands rejected for the recitation "on average, deriving at least 50%" in line 2 that renders the claim indefinite.

Applicant has now cancelled claim 38, alleviating this rejection.

The Examiner rejects claim 39 for the recitation "A 38J53 maize plant selected from the population of 38J54 progeny maize plants" rendering the claim indefinite. The Examiner states the claim is drawn to plant 38J54, yet can comprise less than 100% of the alleles of 38J54.

Applicant has cancelled claim 39, thus alleviating this rejection.

Claim 40 stands rejected as indefinite for the recitation "further comprising applying double haploid methods".

Applicant has now cancelled claim 40, thereby alleviating this rejection.

In light of the above amendments and remarks, Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 11-19 and 24-32 remain and claims 9, 10, 22, 23, 34-40 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention, for the reasons of record stated in the Office Action mailed July 29, 2002. The Examiner states that the deposit of seed of plant 38J54 does not provide a description of the plants that are encompassed

by the rejected claims, which have not been deposited. The Examiner also states that no description of any trait is provided concerning the other parents of the claimed plants or of the 50% of the alleles of the claimed plants which will be inherited from the other parent. The Examiner suggests that claims 12 and 25 be amended by listing the type of transgenes that may be introduced. Finally the Examiner concludes that the specification does not describe any traits of any inbred plants or any progeny plants produced from 38J54, nor does it mention any double haploid method.

Applicant acknowledges the written description rejection to claims 8 and 21 as withdrawn. Applicant has now cancelled claims 9-19, 21-32 and 34-40, thus alleviating this rejection. Applicant has added new claims 41-61, placed in the format suggested by claims faxed by Supervisory Patent Examiner Amy Nelson on August 2, 2002 and again on November 15, 2002 by Examiner David Fox. Applicant believes the new claims come within the purview of the written description requirement and do not add new matter.

Applicant respectfully asserts the following regarding double haploid breeding. The specification discusses multiple breeding techniques that may be used according to the invention. The specification at page 3 states "[p]lant breeding techniques known in the art and used in a maize plant breeding program include, but are not limited to, recurrent selection backcrossing, pedigree breeding, restriction length polymorphism enhanced selection, genetic marker enhanced selection and transformation" (page 3, specification). Double haploid breeding is a technique long known and used in the art of plant breeding. Applicant is attaching herewith Wan *et al.*, "Efficient Production of Doubled Haploid Plants Through Colchicine Treatment of Anther-Derived Maize Callus", Theoretical and Applied Genetics, 77:889-892, 1989. This demonstrates that haploid breeding is a long known technique in the art of plant breeding and supports Applicant's assertion that producing double haploids is well known to one ordinarily skilled in the art. It is axiomatic in patent law that a specification need not include, and preferably omits, what is well known in the art. See *In re Myers*, 161 U.S.P.Q. 668 (CCPA 1969). Double haploids are produced by the doubling of a set of chromosomes (1N) from a heterozygous plant to produce a completely homozygous individual. This is advantageous because the process can eliminate the generations of selfing needed to obtain a homozygous plant from a heterozygous source. Therefore, Applicant respectfully submits that the new claims 46 and 54 comply with 35 U.S.C. § 112, first paragraph.

Claim 33 stands rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, for the reasons of record stated in the Office Action mailed July 29, 2002 for claims 1-32. Applicant's arguments have been fully considered and found persuasive for claims 1-32. The Examiner states the claim is drawn towards a method of making a hybrid plant designated 38J54 comprising crossing inbred maize plant GE500988 and GE533415. The Examiner states that claim 33 recites the deposit numbers for the two inbred maize plants, and page 7 of the specification indicates that these lines have been deposited yet the Examiner states the terms of the deposits are not known.

Applicant respectfully traverses this rejection. Applicant respectfully submits that an actual ATCC deposit has been made for the inbred parents GE500988 and GE533415 as indicated in the October 29, 2002 Amendment. In addition the specification was amended on page 7 to include the ATCC deposit numbers and dates of deposit of both inbred parent plants. Further, Applicant has now amended the DEPOSITS section on page 42 to further include the inbred parents GE500988 and GE533415. The Applicant provides assurance that:

- a) during the pendency of this application access to the invention will be afforded to the Commissioner upon request;
- b) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent;
- c) the deposit will be maintained in a public depository for a period of thirty years, or five years after the last request for the enforceable life of the patent, whichever is longer;
- d) a test of the viability of the biological material at the time of deposit will be conducted (see 37 C.F.R. § 1.807); and
- e) the deposit will be replaced if it should ever become inviable.

Therefore, Applicant submits at least 2500 seeds of hybrid maize plant 38J54 and inbred parent plants GE500988 and GE533415 have been deposited with the ATCC. In view of this assurance, the rejection under 35 U.S.C. § 112, first paragraph, should be removed. (MPEP § 2411.02) Such action is respectfully requested.

In light of the above amendments and remarks, Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph.

Applicant acknowledges that claims 1-5, 7 and 20 are allowed.

Conclusion

In conclusion, Applicant submits in light of the above amendments and remarks, the claims as amended are in a condition for allowance, and reconsideration is respectfully requested.

No additional fees or extensions of time are believed to be due in connection with this amendment; however, consider this a request for any extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Reconsideration and allowance is respectfully requested.

Respectfully submitted,



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Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus

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Summary. A chromosome doubling technique, involving colchicine treatment of an embryogenic, haploid callus line of maize (*Zea mays* L., derived through anther culture), was evaluated. Two colchicine levels (0.025% and 0.05%) and three treatment durations (24, 48, and 72 h) were used and compared to untreated controls. Chromosome counts and seed recovery from regenerated plants were determined. No doubled haploid plants were regenerated from calli without colchicine treatment. After treatment with colchicine for 24 h, the callus tissue regenerated about 50% doubled haploid plants. All of the plants regenerated from the calli treated with colchicine for 72 h were doubled haploids, except for a few tetraploid plants. No significant difference in chromosome doubling was observed between the two colchicine levels. Most of the doubled haploid plants produced viable pollen and a total of 107 of 136 doubled haploid plants produced from 1 to 256 seeds. Less extensive studies with two other genotypes gave similar results. These results demonstrate that colchicine treatment of haploid callus tissue can be a very effective and relatively easy method of obtaining a high frequency of doubled haploid plants through anther culture.

Key words: *Zea mays* – Anther culture – Embryogenic haploid callus – Chromosome doubling

Introduction

The success of producing haploid plants in maize through anther culture makes it possible to generate

inbred lines through chromosome doubling (Kao et al. 1986). However, the application of anther culture to plant breeding is largely dependent on the production of large numbers of haploid plants and the high frequency of induction of chromosome doubling. In maize, anther-derived lines have been developed and used commercially (Wu et al. 1983). However, the frequency of chromosome doubling of anther-derived haploid plants either spontaneously or through colchicine treatment has been undesirably low (Ku et al. 1978; Nitsch et al. 1982; Miao et al. 1978). Ku et al. (1978) and Nitsch et al. (1982) observed only 6.3% and 4.5% spontaneously doubled haploids among plants regenerated from cultured maize anthers, respectively. Miao et al. (1978) treated anther-derived plantlets and obtained only one plant which set seeds from the 24 plants that survived.

With many plant species, chromosome doubling can be achieved by the use of an antimutagenic agent treatment of anther-derived, haploid plantlets. Since antimutagenic agents such as colchicine usually induce chromosome doubling in only some cells due to the asynchrony of cell divisions, chimeric plants are common after colchicine treatment. For plant species which produce bisexual flowers and tillers or branches, chimeras are acceptable since some tillers or branches may develop from the chromosome-doubled cells. In contrast, maize plants usually do not produce tillers and cell lines which give rise to the tassel and ear are already determined in the mature seed (Coe and Nouffer 1978). Colchicine treatment of maize seedlings or plantlets may double the chromosome number in the tassel or ear, but often not in both, which will make self-pollination impossible. These reasons may explain why the efficiency of inducing doubled haploid plants in maize is very low by colchicine treatment of regenerated haploid plantlets (Miao et al. 1978).

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Since somatic embryos from tissue cultures may develop from one or a few cells, it may be possible to induce chromosome doubling in embryogenic haploid callus and then induce plant regeneration from this tissue (Genovesi and Collins 1982). The use of a long term haploid culture system capable of plant regeneration may make the chromosome doubling technique effective as proposed by Tsai et al. (1986). This paper reports the recovery of doubled haploid plants with high frequency through colchicine treatment of embryogenic haploid callus initiated from maize anther culture.

Materials and methods

Establishment of callus cultures. F1 plants of a maize hybrid, H99 × P16, were grown in the field in 1987. Tassel collection to anther plating were carried out by previously described methods (Petolino and Thompson 1987). Petri dishes containing anthers were placed in plastic boxes covered with aluminum foil at 28°C. About 1 month later, embryo-like structures began to appear from responding anthers. The embryo-like structures were removed from the anthers and were transferred to a callus induction medium. The callus induction medium consisted of macronutrients and vitamins of N6 medium (Chu et al. 1973), micronutrients of B5 medium (Gamborg et al. 1968) with 2,4-D (0.45 µM), dicamba (11.3 µM), myo-inositol (0.35 mM), L-proline (25.0 mM), caseinatic casein hydrolysate (0.1 g/l), sucrose (87.6 mM), Na₂EDTA (116.55 µM) and FeSO₄ · 7H₂O (100.2 µM). Callus lines, each of which was derived from a single embryo-like structure, were maintained in the callus induction medium through subcultures by selective transfer of the embryogenic calli at 4-week intervals. One highly regenerable callus line was used 6 months after culture initiation.

Colchicine treatment. Colchicine was dissolved in water to make a stock solution which was filter-sterilized and then added to liquid D medium (Duncan et al. 1985) to the required final concentrations and stored in the dark. About 20 ml of the medium was placed in Petri dishes (100 × 25 mm) and a filter paper disc supported by a stainless steel screen, which were autoclaved previously, was saturated with the liquid medium. Embryogenic calli, 20 days after subculture, were cut into 0.5–1.0 mm pieces and were plated on the moist filter paper and incubated in the dark at 28°C. Following treatment, the calli were placed on a stainless steel screen and were rinsed twice in liquid D medium without colchicine. Two colchicine levels (0.025% and 0.05%) and three treatment durations were used and compared with untreated control.

Plant regeneration from treated calli. Colchicine treated calli were subcultured two times with an interval of 10 days on agar-solidified D medium. For plant regeneration, calli were transferred to H medium (Duncan et al. 1985) with 3.5 mg/l 6-benzyladenine for 3 days. The calli were then cultured in H medium until some regenerated plantlets grew to 3–4 cm long, which occurred within about 20 days. The regenerated plantlets were transferred to H medium minus R₁ vitamins and glucose in culture tubes for further growth. After 7–10 days, they were transplanted to soil in 11.5-cm pots and grown for another 10–15 days (or even longer depending on the growth of each plant). Finally, the plants were transplanted to 27.5-cm pots in the greenhouse and at least two root tips were collected from each plant for mitotic examination.

Plants with pollen and silks were self-pollinated on successive days. The dates of the first day of pollen shed and the first day of silk emergence were recorded for 33 representative doubled haploid plants. Seeds were harvested 40–45 days after pollination.

Determination of ploidy level. The root tips were cold-treated in ice water for 24 h and fixed in 3:1, 95% ethanol:glacial acetic acid for 24 h and then stored in 70% ethanol. For mitotic examination, the root tips from each plant were placed in a small vial with 1% aceto-carmum and heated to the boiling point several times. The meristematic region was excised and squashed in one drop of 45% glacial acetic acid on a slide. At least two root tips from each regenerated plant were examined to determine the ploidy level.

Results

All 24 plants regenerated from the untreated calli contained the haploid number of ten chromosomes (Table 1, Fig. 1a). Of 96 plants regenerated from calli treated for 24 h with either 0.025% or 0.05% colchicine, 49 were diploid with 20 chromosomes (Fig. 1b), and the other 47 were haploid with ten chromosomes. Of 53 plants from the calli treated with colchicine for 48 h, 29 were diploid plants. Calli treated for 72 h did not regenerate any haploid plants, with most being diploid plants except for one and four tetraploid plants with 40 chromosomes obtained from the two 72-h treatments of 0.025% and 0.05% colchicine, respectively. No significant difference in chromosome doubling was observed between these two colchicine levels (Table 1).

The haploid plants regenerated in this study all displayed a characteristic morphology (short, narrow leaves, reduced vigor, and no pollen shed). Under the same growing conditions, the doubled haploid plants

Table 1. Ploidy of plants regenerated from colchicine-treated haploid calli as determined from root tip squashes

Hours	Concentration	No. of plants regenerated			
		Total	Haploid	Diploid	Tetraploid
24	-	24	24	0	0
	0.025%	48	23	25	0
	0.05%	48	24	24	0
Total		96	47	49	0
48	0.025%	22	8	14	0
	0.05%	31	16	15	0
Total		53	24	29	0
72	0.025%	31	0	30	1
	0.05%	32	0	28	4
Total		63	0	58	5

were generally more vigorous in appearance and grew more rapidly when compared with the haploid plants (Fig. 2). The doubled haploid plants from different treatments exhibited similar morphology. Most of them produced abundant, viable pollen. A common feature of many of the doubled haploid plants was the appearance of tassels with some female flowers. The ears of these plants could, however, still be self-pollinated if the silks emerged in time.

Most of the doubled haploid plants, 107 of 136, produced from 1 to 256 seed per ear after self-pollination. A few ears had almost normal seed set (Fig. 3). Among 29 doubled haploid plants which did not produce seed, 21 of the plants could not be pollinated due to asynchronous pollen shed and silk emergence, the lack of ear development, or to stunted growth. Eight other plants produced no seed even after being pollinated one or two times on successive days. The synchrony of pollen shed and silk emergence were the main factors which affected the seed production by the doubled haploid plants. As shown in Table 2, if the silks emerged for pollination 1–3 days later than the first pollen was shed, an average of more than 87 seeds per ear were set. If the pollination was started 4 days later than the first pollen was shed, the seed set was dramatically decreased to 39 seeds per ear. Most plants would not set seed if silk emergence was delayed 5 days or more after pollen shed began.

Five tetraploid plants were found among the plants regenerated after the two 72-h colchicine treatments. Of these five plants, two plants had terminal ears and three plants had good pollen shed, but due to late silk emergence, only two of the plants produced one seed each after self-pollination.

Anther-derived callus lines from two other hybrids, H99 × Pa91 and Pa91 × Fr16, were also treated with colchicine. Due to lower regenerability of the callus line from Pa91 × Fr16 and incomplete experiment design for the callus line from H99 × Pa91, the data from these two lines are not included. However, these experiments also showed that the longer the callus cultures were incubated

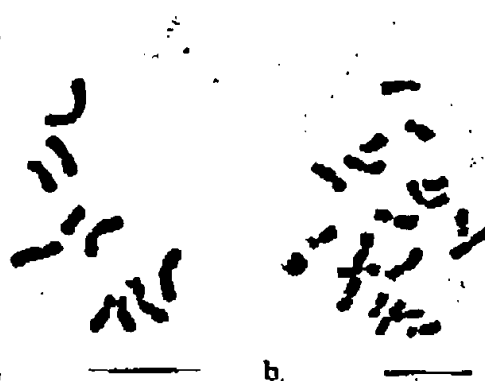


Fig. 1a and b. Root tip chromosomes from a haploid cell with 10 chromosomes from a plant regenerated from untreated callus and b a diploid cell with 20 chromosomes from a plant regenerated from colchicine treated callus. Bar represents 10 μ m



Fig. 2. Typical appearance of a doubled haploid plant (middle) from colchicine treated haploid callus, haploid plant (right) from colchicine-treated haploid callus, and haploid plant (left) from untreated callus. The pot diameters are 27.5 cm



Fig. 3. Mature ears resulting from self-pollination of some doubled haploid plants

Table 2. The relationship between the delay in silk emergence after the beginning of pollen shed and the average number of seed produced per ear from 33 randomly selected doubled haploid plants

Silk emergence delay (d)	No. of plants pollinated*	Average seeds per ear	No. of ears without seed
1	4	100.3	0
2	8	91.0	0
3	8	87.1	0
4	5	39.0	0
> 5	8	2.5	5

* One ear per plant was self-pollinated

in colchicine-containing medium, the more diploid plants were regenerated, and no diploid plants were regenerated from the control calli without colchicine treatment. These results then indicate that chromosome doubling of maize callus tissue by colchicine treatment is reproducible and is not genotype-specific.

Discussion

The present study shows that the colchicine treatment of the embryogenic haploid maize callus can be very effective for producing a large number of doubled haploid plants. By incubating embryogenic haploid calli on colchicine-containing medium, doubled haploid plants were produced at high frequencies. Since all the plants from untreated calli were haploids, the occurrence of doubled haploid plants must be due to the effect of colchicine. The method is rapid since it only required 6 months from colchicine treatment of calli to the harvest of seeds from the regenerated doubled haploid plant.

The results of this study suggest that the duration of colchicine treatment is important. The treatment of more than 48 h is necessary in order to get higher frequency of doubled haploids among the regenerated plants. If the treatment is 72 h, tetraploid plants could be produced, which may not be desirable. The two concentrations of colchicine used, 0.025% and 0.05%, did not show significant differences in their chromosome doubling efficiency.

There was no indication that ploid chimeras were regenerated, since most of the doubled haploid plants produced seeds after self-pollination. The problem which caused the doubled haploid plants to not set seeds was mainly delayed silking emergence or the lack of ear formation, which are common phenomena among tissue culture-derived maize plants (Miao et al. 1978; Petolino and Jones 1986). The abnormal plants found among the regenerates were probably due to the tissue culture conditions rather than the colchicine treatment since the same abnormalities (stunted growth, terminal ear, the lack of normal ear) existed among the plants regenerated from untreated control calli. In practice, only vigorous plantlets should be selected before transplanting to greenhouse or field. This should reduce the frequency of abnormal plants.

The results show that colchicine treatment of embryogenic haploid callus can result in the production of

entire doubled haploid plants with high frequency, which produce fertile maize inbred lines within a short time at a high frequency, thus making the anther culture technique more useful to the plant breeder.

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